

Purification and characterization of the *in vitro* activity of I-Sce I, a novel and highly specific endonuclease encoded by a group I intron

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ABSTRACT

Group I intron encoded proteins represent a novel class of site specific double strand endonucleases. The endonuclease activity of this class of proteins has been first demonstrated *in vivo* for I-Sce I which is encoded by a mitochondrial intron of *Saccharomyces cerevisiae*. Assays using crude cell extracts have shown that I-Sce I can be used *in vitro* as a restriction endonuclease potentially useful for recombinant DNA technology owing to its large recognition sequence (18 nucleotides). We report here the purification and the first detailed analysis of the *in vitro* activity and properties of I-Sce I.

INTRODUCTION

It has recently been recognized that a number of group I introns of various organisms exhibit genetic mobility in crosses (reviewed in 1). Such introns encode double strand endonucleases that cleave intron-less genes in a sequence specific manner. In each case, the intron propagates itself by insertion into the double strand cut generated by the action of its endonuclease. The insertion is associated with the coconversion of flanking exons in a manner consistent with the mechanism of double strand break repair as proposed for yeast.

The optional group I intron of the mitochondrial LSU gene of *Saccharomyces cerevisiae* (intron Sc LSU·I) belongs to this category of introns and was, indeed, the first mobile intron to be discovered (2). It contains a 235 codon long open reading frame encoding the endonuclease I-Sce I (formerly called ω -transposase; for recent nomenclature conventions refer to 3). This protein remains below detection level in mitochondria. However, it can be overexpressed in *E. coli* cells after modification of the non-universal codons of the intron ORF (4). The endonuclease I-Sce I is active *in vivo* in *E. coli* cells indicating that it is not the subject of important post-translational modifications in mitochondria. Partially purified fractions from *E. coli* also show that I-Sce I is an active endonuclease *in vitro* conserving the expected specificity (5).

Aside from its interest in the molecular genetics of introns, a most interesting aspect of the I-Sce I endonuclease lies in that its recognition site, which is a non-symmetrical sequence, extends over 18 bp, making it a potentially useful restriction endonuclease with very high sequence specificity. I-Sce I generates a four base pairs staggered cut with 3' OH overhangs (6). We report here the purification, properties and analysis of the *in vitro* enzymatic activity of I-Sce I.

MATERIALS AND METHODS

Plasmids and strains

I-Sce I was purified from JM101 *E. coli* cells transformed by the plasmid pSCM930 as previously described (4). This plasmid contains the universal code equivalent of the intronic ORF, placed under *tac* promoter control.

Buffers

Buffer A₀ was: Tris/HCl 0.05 M, Ethylene diamine tetraacetic acid (EDTA) 0.003 M, phenylmethylsulfonyl fluoride (PMSF) 10⁻⁴ M, glycerol 5% (v/v) at pH 7.5. Buffer H₀ was: Hepes 0.02 M, EDTA 0.003 M, glycerol 5% (v/v) at pH 7.8. Final NaCl concentrations in the previous buffers are indicated by the index (eg. A_{0.2} is buffer A containing NaCl at 0.2 M, H_{0.15} is buffer H containing NaCl at 0.15 M). TBE buffer was: Tris/HCl 0.1 M, boric acid 0.1M and EDTA 0.025 M at pH 8.3.

Extraction procedure

Purifications were routinely carried out using 2 to 4 liters of cultures. *E. coli* cells were grown at 37°C in LBA medium (LB medium containing 50 µg/ml of ampicilline) until the OD₆₀₀ reaches 0.8. Isopropylthio-β-galactoside (IPTG) was then added at a final concentration of 5×10⁻³ M and the cultures were further incubated for 1 to 2 hours. Cells were collected by centrifugation and the washed pellets (7 to 12 g) were frozen to -20°C before extraction (we noticed that pellets can be kept frozen for long periods without significant loss of activity). After thawing, the pellets were redispersed in 30 ml of buffer A_{0.2} and

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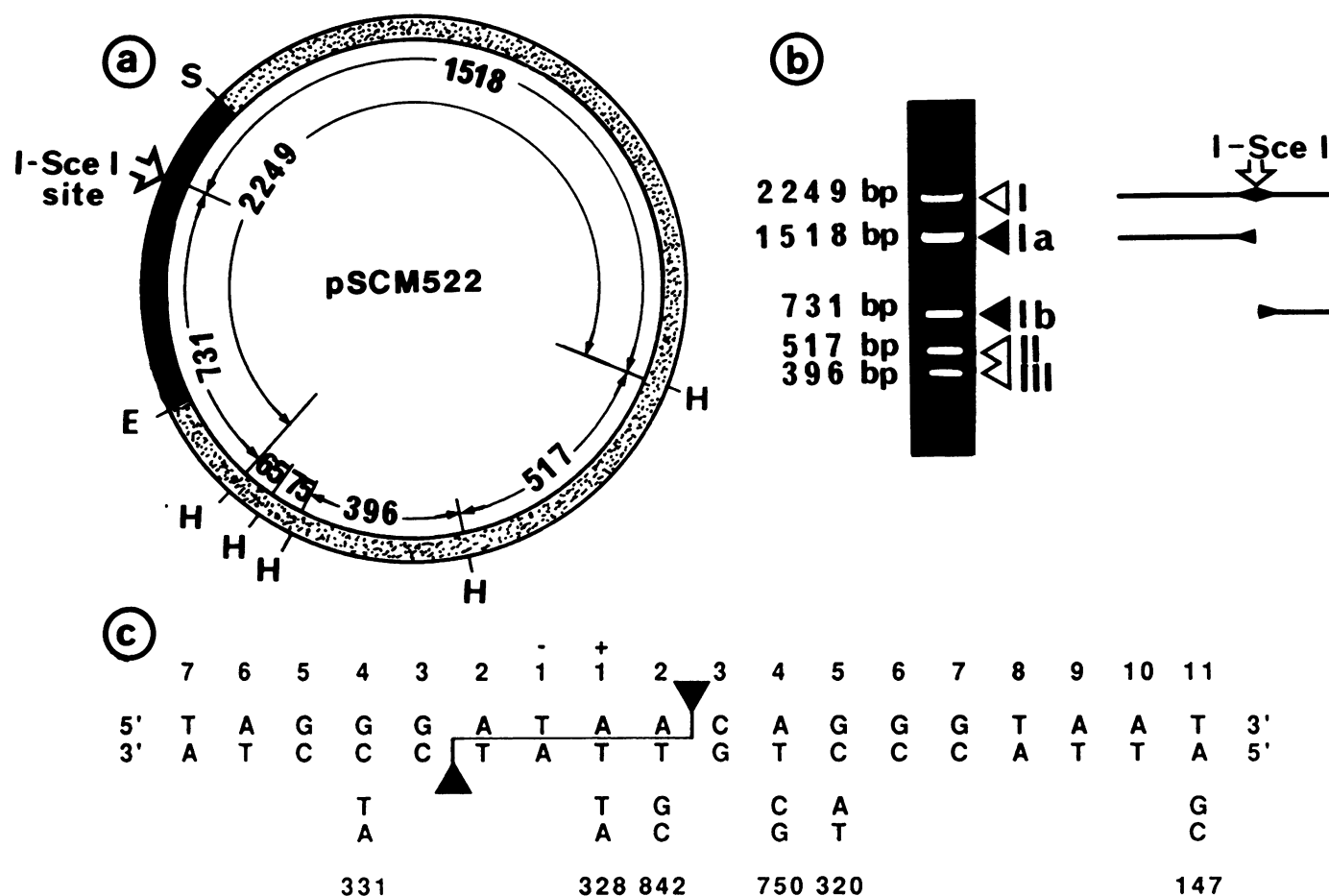



Fig. 1. Standard assays of I-Sce I activity. **Fig. 1a** : pSCM 522 recombinant plasmid (3302 bp) is a pUC 12 derivative containing the I-Sce I recognition site inserted between the Eco RI and the Sal I sites of the polylinker. Sizes of Hinf I fragments are indicated. H = Hinf I ; S = Sal I ; E = Eco RI **Fig. 1b** : Example of cleavage of Hinf I digested pSCM 522 DNA by I-Sce I. Hinf I fragments were numbered I, II and III for 2249, 517 and 396 bp, respectively. Fragment I generates, after cleavage by I-Sce I, two fragments, Ia and Ib, of sizes 1518 and 731 bp, respectively. The % of cleaved product is expressed by *R*, the ratio of peaks area corresponding to cleaved fragments Ia and Ib over peaks area corresponding to fragments I, Ia and Ib. ($R = 100 \times (Ia + Ib) / (I + Ia + Ib)$) **Fig. 1c** : Recognition site of I-Sce I and definition of mutants used in this work (data from 6). Numbering is from the center of the staggered cut ().

incubated on ice with 1mg/ml lysozyme for 30 to 60 minutes. After lysis, 20 to 30 ml of buffer $A_{0.2}$ and 1 μ l of antifoaming agent (Sigma A-5758) were added to the incubation mixtures which were then homogenized in a blender at 2°C for 100 seconds in total (pulses of 10 seconds separated by 30 seconds arrest periods were applied to reduce heating). Extracts were centrifuged twice at 20000 RPM for 30 minutes at 2°C to eliminate cell debris and insoluble material. Supernatants were loaded onto a 70ml Trisacryl-QAM column (Industrie Biologique Française) equilibrated with buffer $A_{0.2}$ at 2°C. This column was eluted with the same buffer, at a flow rate of 1.5 ml/minute and 6 ml fractions were collected. On-line UV monitoring of chromatographies was performed using Pharmacia Dual Path monitor UV2. The I-Sce I activity was found in the flowthrough. Pools of active fractions (100 to 150 ml) were dialysed against buffer $H_{0.25}$ containing 15% (w/v) polyethylene glycol (35 000 MW) at 0°C until the volume was reduced approximately 4 folds. The concentrated solutions were then loaded onto a 30 ml S-sepharose column (Pharmacia) also equilibrated with buffer $H_{0.25}$ at 2°C. This column was eluted with buffer $H_{0.25}$ at a flow rate of 1ml/min until the U.V. absorbance diminished to approximately 30% of the initial value. The eluting buffer was

then changed to $H_{0.5}$ and fractions of 4ml were collected. Most I-Sce I activity was found in the fractions eluting at 0.5M NaCl. These fractions were pooled, dialysed against buffer H_0 at 2°C, then loaded again onto the 30 ml S-sepharose column equilibrated with buffer H_0 . The column was eluted with increasing NaCl concentrations steps of 0.1M, starting with $H_{0.15}$ up to $H_{0.45}$, at a flow rate of 1ml/minute. Four ml fractions were collected. I-Sce I activity was present in the fractions eluted from 0.35M NaCl to 0.45M NaCl. Active fractions were pooled and concentrated to approximately 5ml by dialysis against 15% polyethylene glycol in buffer H_0 as above. Concentrated aliquots were then dialysed against buffer $A_{0.15}$ and loaded on a 2 ml Q-sepharose column (Pharmacia) equilibrated with buffer $A_{0.15}$. The I-Sce I activity was found in the flowthrough, very little activity remaining bound to the column.

Qualitative assays of activity

Fractions were assayed for I-Sce I activity in the following conditions: 150ng of pSCM 522 plasmid DNA (fig. 1) previously cleaved by Hinf I were incubated with 1 to 2 μ l of the fraction to be tested in a buffer containing diethanolamine/HCl 0.1M pH 9.5, $MgCl_2$ 0.005 M and bovine serum albumin (BSA) at

Table1. Quantitative parameters of the purification strategy employed

Fractionation step	Total protein content (mg)	Total activity (units)	Specific activity (units/mg)	Purification factor	Yield (%)
Crude cell supernatant	730	26000	35.6	1	100
Trisacryl-QAM eluate	140	18000	128.6	3.6	69
S-sepharose eluate	5	6000	1200	34	23
Q-sepharose eluate	1.7	2500	1470	41	10

Fractions were purified from 7.5g of cells (wet weight) according to Material and Methods. Total protein contents have been calculated using the method of Bradford (7). An arbitrary unit of activity is defined as the amount of enzyme needed for complete cleavage at the I-Sce I site of 150 ng of Hinf I digested pSCM 522 DNA (equivalent to 7×10^{-14} moles of recognition site) during a 20 minutes reaction at 37°C in a buffer containing 0.1M diethanolamine/HCl, 0.005 M $MgCl_2$ and 100 $\mu g/ml$ of BSA at pH 9.5 and a total reaction volume of 15 μl .

100 $\mu g/ml$ in a total reaction volume of 15 μl . Reactions were carried out at 37°C for 20 minutes then stopped by addition of 1 μl of EDTA at 0.5 M and analyzed by agarose gel electrophoresis. When crude fractions were tested, 10 μg of proteinase K (Boehringer) was added and assays were left at room temperature for 10 minutes before electrophoresis.

Quantitative measurements of activity

Samples of reactions were analyzed by electrophoresis on 1% agarose gels in TBE buffer. After migration, gels were incubated in a solution of 0.1 $\mu g/ml$ of ethidium bromide (EB) in H_2O with gentle agitation at room temperature for 1hr to ensure homogenous staining. EB fluorescence at 254nm was photographed on Polaroid 665 films. Relative amounts of DNA in the different bands of each sample were then determined from the scanning of the negative films using a Bio-Rad Model 620 video densitometer coupled to a Shimadzu chromatopac C-R3A integrator. Quantitation of I-Sce I activity in each reaction is calculated from the % of cleaved product expressed as in fig.1.

RESULTS

Purification of I-Sce I activity

The purification procedure is given by Table I. Chromatography of crude *E.coli* cell supernatants using Trisacryl-QAM anionic exchange column yields I-Sce I activity in the flowthrough. No significant activity can be recovered after stepwise elutions of the column with buffers $A_{0.4}$ to A_1 . This chromatography eliminates most of the nucleic acids as well as unspecific nucleic acid binding proteins. In the second chromatography, using the S-sepharose cationic exchange column, the I-Sce I activity remains bound to the column in buffer $H_{0.25}$. Elution of this column with buffer $H_{0.5}$ allows the recovery of the activity. Some activity is also found in the fractions eluted with $H_{0.25}$ but these fractions contain contaminating nucleic acids and non specific nucleases which have not been removed by the previous chromatography. On the second passage on S-sepharose with 0.1 M NaCl steps, I-Sce I activity elutes from 0.35 to 0.45 M NaCl. The last chromatography, using Q-sepharose anionic exchange column contributes to the elimination of a few more contaminants. This procedure gives a ca. 40 fold purification of I-Sce I with a 10% recovery. I-Sce I enzyme prepared as above shows specific cleavage activity without significant non specific nuclease degradation of DNA (fig.1b).

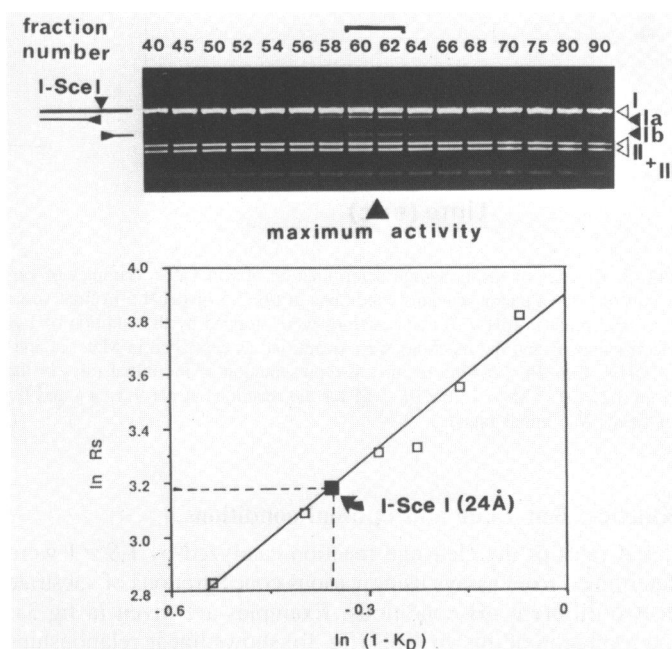


Fig.2. Size-exclusion chromatography of I-Sce I. A 89×2.5 cm Sephacryl-HR 200 column (Pharmacia) was calibrated using aldolase, bovin serum albumin, ovalbumin, β -lactoglobulin, soybean trypsin inhibitor and cytochrome c as standards. Elutions were performed with a flow rate of 0.74 ml/min in buffer $A_{0.15}$ at 2°C and monitored by UV absorbance. A 5 ml active fraction of I-Sce I was then loaded on this column and chromatographed in identical conditions. Elution of I-Sce I was monitored by activity in standard assays (fig 2a). Relationship between the Stokes radii of the standards (taken from 8) and the partition coefficients were represented according to (9) (fig 2b). R_S = Stokes radius in Å; K_D = partition coefficient. Open squares = standard proteins ; full square = I-Sce I

I-Sce I purifies as a monomeric globular protein.

Gel exclusion chromatography was used to determine the size and shape of I-Sce I (fig.2). The activity of I-Sce I in the eluted fractions corresponds, on the plot drawn with the protein standards, to an apparent molecular weight of 26 kD and a Stokes radius of 24 Å. This figure is close to the molecular weight of 27.6 kD calculated from the amino acid composition deduced from the DNA sequence (2), hence I-Sce I appears as a monomeric globular protein.

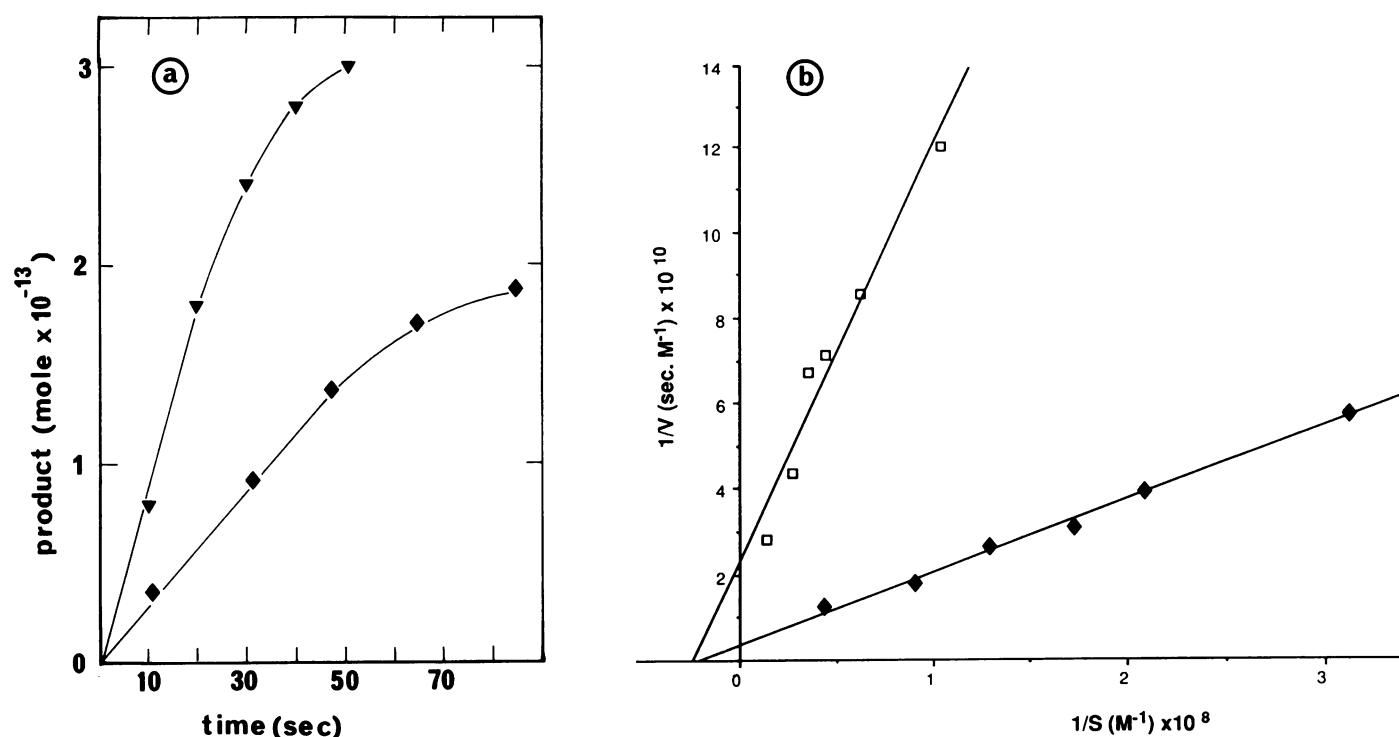


Fig. 3. Kinetics of reactions and determination of K_m value. Samples of various amounts of Hinf I digested pSCM 522 DNA were incubated in the presence of 1 unit of I-Sce I under standard conditions at pH 7.5 or pH 9.5 in final volumes of 66 μ l or 88.5 μ l, respectively. Aliquots were taken at intervals of 10 sec (pH 9.5) or 2 minutes (pH 7.5) and reactions were stopped by the addition of 1 μ l of EDTA at 0.5 M and freezing at -70°C . Aliquots were analyzed by agarose gel electrophoresis and the reactions were quantified as described in Material and Methods. **Fig. 3a** : Examples of kinetics for samples of 0.5 (\blacklozenge) or 2 (\blacktriangledown) picomoles of DNA. **Fig. 3b** : Double reciprocal representation of the initial rates of the cleavage reactions. Initial rates were measured for substrate concentrations varying from 3.2×10^{-9} M to 22.6×10^{-9} M for the reactions at pH 9.5 (\blacklozenge) and from 9.7×10^{-9} M to 77×10^{-9} M for the reactions at pH 7.5 (\square). (S : concentration of sites ; V : initial rates)

Kinetics, K_m value and optimal conditions.

Initial rates of the cleavage reaction catalyzed by I-Sce I were determined from assays using various concentrations of substrate in two different pH conditions. Examples are given in fig.3a. Interpretation of this analysis (fig.3b) shows linear relationships as expected of a Michaelian reaction. K_m values are approximately 4.3×10^{-8} M (pH 7.5) or 3.4×10^{-8} M (pH 9.5) and V_{\max} are 0.4×10^{-10} M/sec. (pH 7.5) or 2.8×10^{-10} M/sec. (pH 9.5).

The effects of temperature, ionic strength and pH have been analyzed to further characterize the *in vitro* reaction and to optimize the assay conditions (fig.4). It appears from our results that the pH has a major effect on the reaction. The endonuclease activity is limited at low pH values and increases rapidly above pH 7 to reach a maximum between 9 and 10. Temperature can be changed from ca. 30°C to 45°C without major effect on the reaction but a significant decrease occurs beyond these limits. Our results also show that the reaction requires no monovalent cations and is even inhibited when the NaCl concentration raises above 0.05M. Magnesium (0.003 to 0.01M) is absolutely required, no reaction takes place in EDTA (data not shown). Manganese in similar concentrations can replace magnesium, although less efficiently. Other divalent cations such as: zinc, calcium, cobalt or copper have been tested but none was found able to replace magnesium.

In vitro stability of I-Sce I

The catalytic activity of I-Sce I in purified fractions can be maintained almost constant for long periods of time (up to 4

months) provided that the enzyme is stored in 50% glycerol solutions at -20°C or simply in the buffer A_0 at 0°C . We noticed, however, that the activity of I-Sce I rapidly decreases during incubation in the reaction buffer at 37°C . We have, therefore, examined the stability of I-Sce I in various preincubation conditions prior to the addition of substrate. Figure 5 shows typical results of such assays. It can be seen that ca. 80% of activity is lost during the first 1–2 minutes of preincubation in the presence of magnesium. In the absence of magnesium, only ca. 50% of activity is lost during the same time. The pH range tested shows little effect in the stability.

Interactions between I-Sce I and mutants of the recognition site

We noticed that the addition to the previous assays of plasmid DNA containing a mutant of the recognition site for I-Sce I contributes to increase the stability of the enzyme. On the contrary, non-specific DNA (eg. pEMBL18+) has no effect (Fig.6). We have analyzed this protection as a means to investigate the interaction between I-Sce I and the mutants of its site. Several mutants of the site have been previously tested for their cleavability by I-Sce I in *in vitro* assays at pH 7.5 (6). Depending upon the position and the nature of the mutation, some of these mutants were completely uncleavable (called 0), others were cleaved as efficiently as the wild type while others were cleaved much less efficiently (called ϵ). In the present study we have reexamined the cleavability of a set of mutants under optimum conditions and, at the same time, their degree of protection on the enzyme (Fig. 7). In each case, the remaining

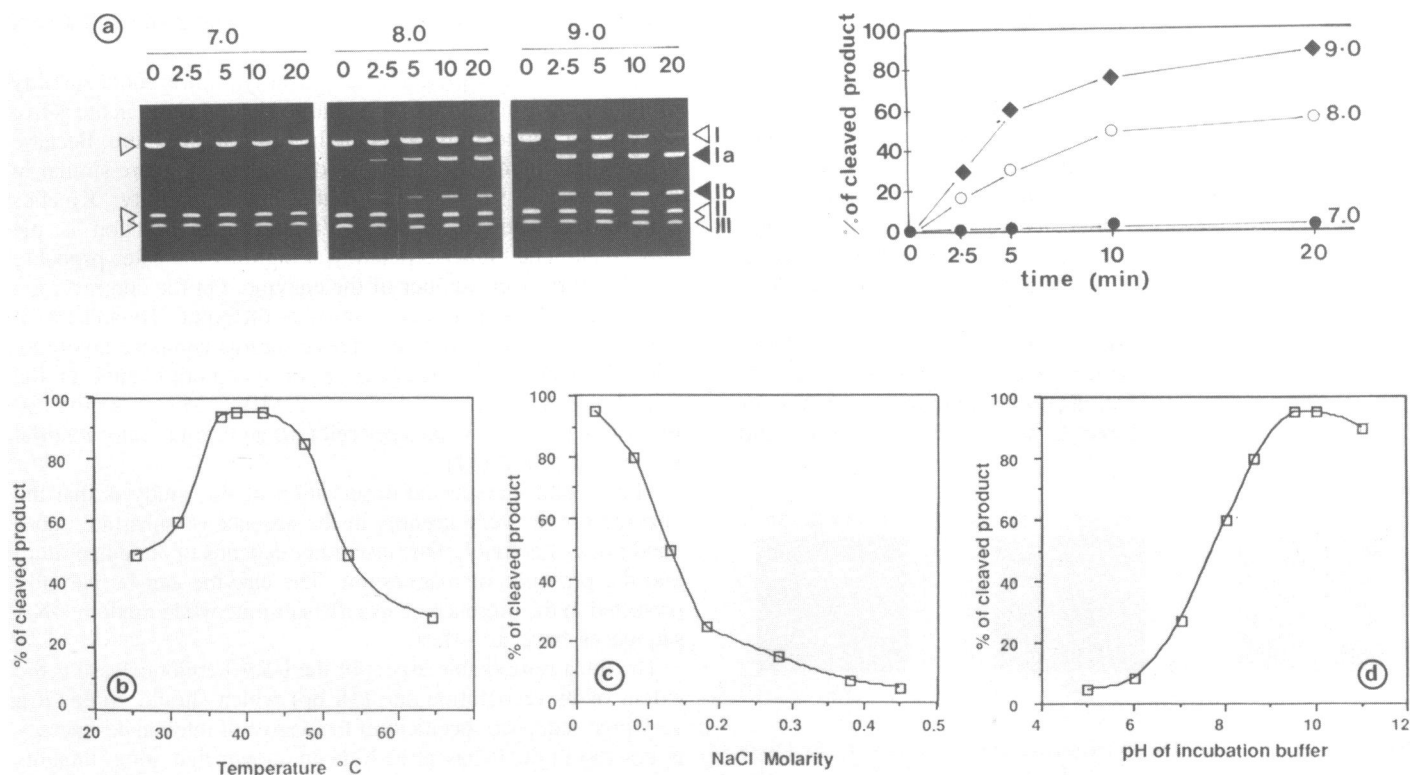


Fig. 4. Determination of optimum conditions. **Fig. 4a** : Kinetics of reaction at various pHs: 1.5 μ g of Hinf I digested pSCM 522 DNA (0.7 picomoles of I-Sce I sites) were incubated with 10 units of I-Sce I in a buffer containing 0.005 M $MgCl_2$ and 0.1 M Tris/HCl at pH 7.0, 8.0 or 9.0 as indicated (total reaction volumes were 100 μ l). Incubation was carried out at 37°C and 20 μ l aliquots were taken at time intervals (times are indicated in minutes). The reactions were stopped by addition of 2 μ l of EDTA at 0.5 M and quick freezing at -70°C . Percent of cleaved product is calculated as in Materials and Methods. **Fig. 4b** : Effect of temperature : 150 ng of Hinf I digested pSCM 522 DNA were incubated with 1 unit of I-Sce I in a buffer containing 0.1 M diethanolamine-HCl at pH 9.5 and 0.005 M $MgCl_2$. Incubations were carried out at various temperatures for 20 minutes. **Fig. 4c** : Effect of ionic strength : 150 ng of Hinf I digested pSCM 522 DNA were incubated with 1 unit of I-Sce I in a buffer containing 0.1 M diethanolamine/HCl at pH 9.5, 0.005 M $MgCl_2$ and various NaCl concentrations as indicated. Incubations were carried out at 37°C for 20 minutes. **Fig. 4d** : Effect of pH : 150 ng of Hinf I digested pSCM 522 DNA were incubated with 1 unit of I-Sce I in a buffer containing 0.005 M $MgCl_2$ and 0.03 M MES (at pH 5.0 or 6.0), 0.1 M Tris/HCl (at pH 7.0, 8.0 or 8.6) or 0.1 M diethanolamine/HCl (at pH 9.5; 10.0 or 11.0) in a total volume of 15 μ l. Incubations were carried out at 37°C for 20 minutes.

enzyme activity is estimated from the % of wild-type substrate cleaved upon completion of the reaction. In such experiments the time 0 (preincubation) represents a direct competition between the wild type and mutant sites since both DNAs were added at the same time. Times 15, 30 and 60 min. indicate simultaneously: -i- the cleavage of mutants sites alone during the preincubation, -ii- the degree of protection of the enzyme by the various mutants sites and -iii- the competition between wild-type and mutants sites after the addition of wild-type DNA. Figure 7a shows the amount of cleavage of mutant sites obtained at the end of reactions. Mutants 320 and 328 are not cleaved, mutants 147 and 331 show little cleavage and mutants 750 and 842 are the most efficiently cleaved. Figure 7b shows the amount of wild type sites cleaved at the end of the reaction after various preincubation times. As already demonstrated, the enzyme alone is extremely unstable. Presence of mutants increases the stability of the enzyme to various degree, 842 being the most effective.

DISCUSSION

In this paper we have described a protocol for the isolation of active fractions of I-Sce I, a new endonuclease encoded by a group I intron and responsible *in vivo* for the cleavage of intron-less genes resulting in the insertion of the intron into the cleaved site.

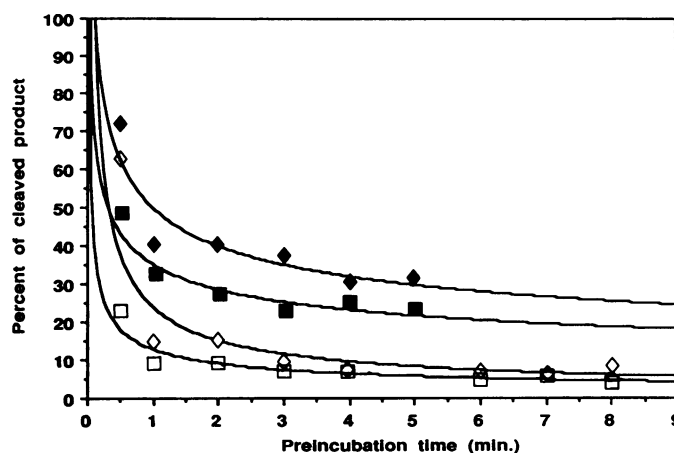


Fig.5. Spontaneous inactivation of I-Sce I. 10 units of I-Sce I were incubated at 37°C in a buffer containing 0.1 M Tris/HCl (at pH 7.0 or 9.0) with or without 0.01M $MgCl_2$ in total sample volumes of 120 μ l. Aliquots of 15 μ l were taken at various time intervals and added with 15 μ l samples containing 150 ng of Hinf I digested pSCM 522 DNA in 0.1 M Tris/HCl pH 9.0 with or without 0.01 M $MgCl_2$ ($MgCl_2$ is added if absent from preincubation). Incubation was carried out at 37°C for another 30 minutes and reactions were stopped by addition of 1 μ l of EDTA at 0.5 M and freezing at -70°C . (♦) pH 9 without magnesium; (◇) pH 9 with magnesium; (■) pH 7 without magnesium and (□) pH 7 with magnesium

The demonstration that this protein is endowed with endonuclease activity was first obtained by *in vivo* assays in *E. coli* (4). We have now demonstrated that the enzyme is active *in vitro*, showing that it is sufficient by itself to recognize a specific DNA sequence and catalyze a double strand break within the recognition site determined previously (6). This work represents the first enzymatic characterization of an intron encoded nuclease.

Our procedure allows the rapid preparation of several thousands units of enzyme from a relatively small quantity of *E. coli* cells. Active fractions show no significant contamination by non-specific nucleases under the conditions described and, therefore, can be used to cleave DNA in a clean and reproducible fashion. No cleavage occurs on sequences other than the recognition site.

Upon systematic examination of the optimum conditions for the *in vitro* activity of I-Sce I, we observed a strong and

unexpected pH dependence of the reaction with optimum for very high pH values.

This may be interpreted in several ways: differential stability of the enzyme, involvement of a charged lateral chain in the active site or direct effect of high pH on the substrate structure. Because in our experiments the stabilities of the enzyme were similar at pH 7 and pH 9, we exclude the first possibility. Kinetics experiments showed that the V_{max} of the reaction is pH dependant. The higher efficiency at high pH indicates probably a higher turn over number of the enzyme. On the contrary, the apparent K_m values remain similar at different pHs and are of the order of 3 to 4×10^{-8} M. These figures compare favorably with the estimated concentration of recognition sites in the mitochondria *in vivo* which is 8×10^{-7} M, assuming 30 mitochondrial DNA copies per cell (10) and a total mitochondrial volume of $5 \mu m^3$ (11).

The second unexpected observation of this study is that the enzyme proves very instable in the absence of substrate under conditions of activity. This instability depends upon temperature and the presence of magnesium. The enzyme can be partially protected in the presence of specific substrate while random DNA sequences have no effect.

The most remarkable aspect of the I-Sce I endonuclease is the extent of its recognition site (18 bp) which should confer it a very high sequence specificity if the degree of internal degeneracy is not too high. It has already been shown that some mutants in the recognition site are tolerated which only partially affect the cleavage reaction at pH 7.5. Our results with a purified fraction now confirm and extend this observation in the optimum *in vitro* conditions. The present protection experiments also show that some mutants, although uncleavable or poorly cleavable, bind to the enzyme and increase its stability. Binding constants of the mutants would be interesting to determine in the future.

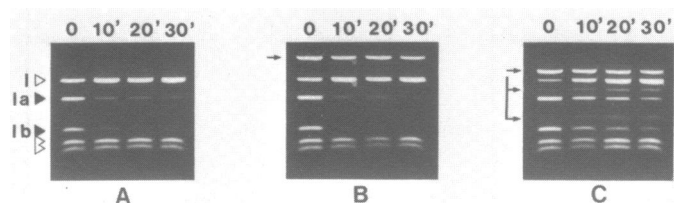


Fig. 6. Protection of I-Sce I by its recognition site. Three units of I-Sce I were incubated at 37°C in Tris/HCl buffer (pH 9.0) 0.1 M ; $MgCl_2$ 0.005 M, during various times (from 0 to 30 minutes) in the absence of DNA (A), in the presence of 400 ng of pEMBL 18⁺ DNA (B) or in presence of 400 ng of a pUC 13 recombinant clone containing mutant 331 (C) in a total volume of 80 μ l. Aliquots of 20 μ l were taken at different time intervals, added with 150 ng of Hinf I digested pSCM 522 DNA and incubated at pH 9.0 and 37°C during 30 minutes. Reactions were stopped and analyzed as described in Materials and Methods. Times are indicated in minutes. (\triangleright and \blacktriangleright) : Fragments of pSCM 522 DNA; (—) : Fragments of pEMBL 18⁺ or mutant 331.

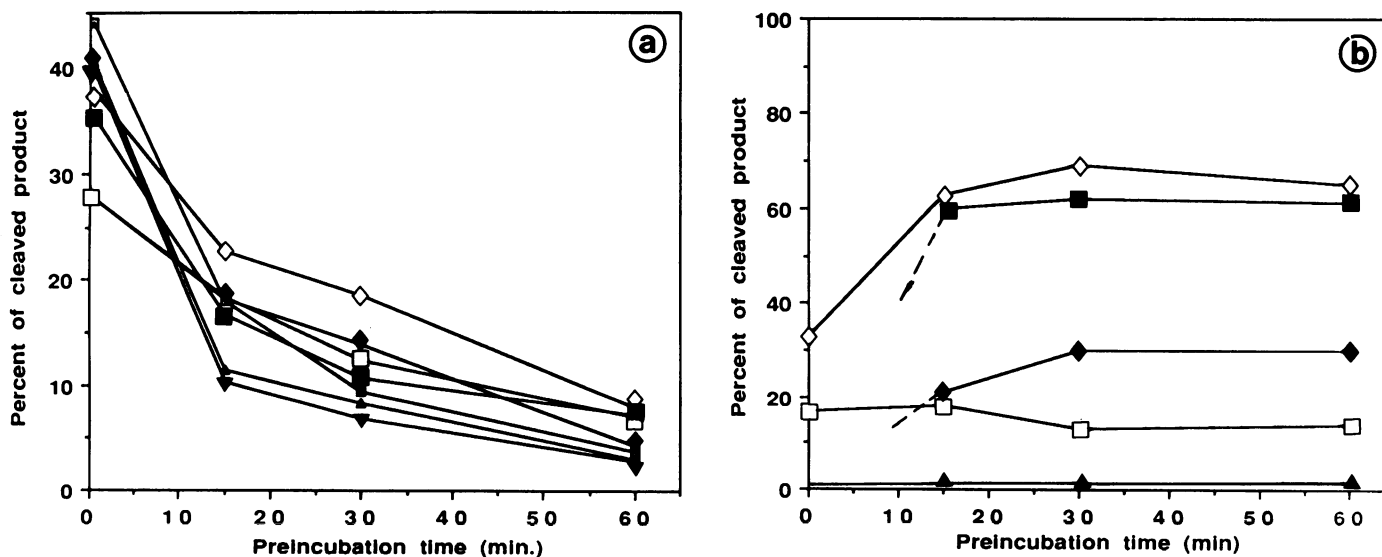


Fig. 7. Interactions of I-Sce I with various mutants of the recognition site. Three units of I-Sce I were incubated at 37°C in a buffer containing 0.1 M Tris/HCl pH 9.0; 0.005 M $MgCl_2$ in the absence of DNA or in presence of 200 to 300ng of *Sca*I digested pUC 13 recombinant plasmids containing mutants of the recognition site in a total volume of 80 μ l. Aliquots of 20 μ l were taken at various time intervals (0, 15, 30 and 60 minutes) and added to 2 μ l containing 150ng of Hinf I digested pSCM 522 DNA in 0.1 M Tris/HCl pH 9.0. Incubations were carried out at 37°C during 30 minutes. Reactions were stopped and analyzed as described as in Materials and Methods. **Fig. 7a** : Cleavage of mutants sites by I-Sce I : The graph represents the extent of cleavage of the different mutants DNA (see fig. 1), after incubation with wild-type DNA as described above. **Fig. 7b** : Residual activity of I-Sce I : The graph shows the residual activity on the wild-type site observed after incubation of I-Sce I in the absence of DNA (\blacktriangledown) or in the presence of various DNAs mutants of the site: (\diamond) : 842; (\blacksquare) : 750; (\blacklozenge) : 331; (\square) : 147; (\square) : 328 and (\blacktriangle) : 320.

Other potential activities of I-Sce I, suggested by its role *in vivo*, have been tested using the purified fractions. Neither any exonucleolytic activity on the cleaved substrate nor any recombinase activity between two cleaved sites or between one cleaved site and uncleaved homologous DNA could be detected *in vitro*. In addition, no double strand cleavage activity could be found on the exon-intron junctions, although each sequence contains half of the recognition site (5). We have also tested the action of I-Sce I on single strand DNA containing the recognition site and found no activity. The absence of activities other than the site specific endonuclease supports the notion that the role of I-Sce I (and probably other group I intron encoded endonucleases as well) *in vivo* is simply to introduce a double strand break in the intron-less gene. Subsequent steps leading to intron insertion necessitate, therefore, the action of other proteins, possibly involved in a general double strand break repair mechanism in mitochondria.

I-Sce I is the first group I intron encoded endonucleases the *in vitro* activity of which has now been characterized in some details. Recently, the activity of five other group I intron encoded endonucleases have been reported based on *in vivo* assays, cell extracts or *in vitro* synthesized proteins: I-Sce II encoded by the yeast intron Sc coxI·4 (12, 13), I-Tev I and I-Tev II encoded by two introns of bacteriophage T4 (14, 15, 16), I-Ceu I encoded by intron Ce LSU·5 of *Chlamydomonas* chloroplast (17) and I-Ppo I encoded by the nuclear group I intron Pp LSU·3 of *Physarum* (18). So far, data on the *in vitro* properties of such endonucleases are only available for I-Tev I (16). Although not systematically examined, the properties of this last enzyme seem to differ from those of I-Sce I. However, I-Tev I belongs to a group of proteins different from that of the endonucleases of chloroplast or mitochondrial origins. Those, although not closely related in terms of their primary amino acid sequences or in terms of their recognition sites, share common structural motives (dodecapeptides or LAGLI-DADG). In addition, all endonucleases from this last group generate four base pair staggered cuts with 3'OH overhangs (reviewed in 1). The same properties are also shared by HO, the nuclear encoded yeast endonuclease responsible for mating type switching (19, 20). Although not of intron origin, that endonuclease also possesses the dodecapeptide motif and has a non symmetrical recognition site which extends over 18 bp.

A major limitation in the use of I-Sce I as a restriction enzyme is the relatively low yield of our purification procedure (assuming a 1 to 1 molar ratio of enzyme to substrate, 2500 units—the total preparation—represent 4.7 μ g of the protein, and it is clear that the actual ratio is much below this value). Part of this low yield results from the low production of the enzyme by *E.coli*, the other from the probable instability of the enzyme in *E.coli*, as inferred from its properties *in vitro*. We have constructed other recombinant plasmids to try to solve the first problem. Experiments are now in progress with such constructions.

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